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Research Article

A new method for screening and determination of diuretics by on-line CE-ESI-MS

A rapid, high-resolution and effective new method for analyzing 12 diuretics by CE-ESI-MS was established in this paper. Ten diuretics (except two neutral compounds) could be fast separated by CE with a DAD at 214 nm with a 20 kV voltage within 6 min, using a 50 μm id and 48.5 cm effective length uncoated fused-silica capillary in a 40 mM ammonium formate buffer (pH 9.40). CE was coupled to the mass spectrometer applying an orthogonal electrospray interface with a triple-tube sheath liquid arrangement. The sheath liquid was composed of isopropanol-water (1:1 v/v) containing 30 mM acetic acid with a flow rate of 4 μL/min. Mass spectrum was employed in the positive mode and both full scan mode and SIM scan mode were utilized. All 12 diuretics could be detected and confirmed by MS in a single analysis. Under optimized conditions, LODs for the 12 diuretics were in the range of 0.13–2.7 μmol/L at an S/N of 3, and the correlation coefficients R^2 were between 0.9921 and 0.9978. The RDSs ($n = 5$) of the method was 0.24–0.94 % for migration times and 1.6–8.8 % for peak areas. The recoveries of spiked samples of 12 diuretics were between 72.4% and 118%. The real urine samples were injected directly for analysis, with only simple filtration through a 0.22 μm membrane filter in order to remove solid particles, which may cause capillary blockage. Based on the migration times and characteristic ions, the diuretics in urine samples were detected successfully. This CE-ESI-MS method for analyzing diuretics will hopefully be applied to doping control.

Keywords:
CE-ESI-MS / DAD / Diuretics / Urine
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1 Introduction

Diuretics are the agents that promote the excretion of urine through their effects on kidney function. They are widely used in clinical practice mainly in the treatment of congestive heart failure and hypertension [1]. Diuretics were misused in sports competition, and have been forbidden by the Medical Commission of the International Olympic Committee since 1988 for the following two reasons. First, athletes may wish to reduce their body weight quickly in order to qualify for a lower mass class. Second, athletes may wish to dilute their urine to avoid a positive doping result by increasing the amount of urine excreted. Misused diuretics will cause a lack of potassium and generate metabolic disorders, which could result in some further diseases [1, 2].

Diuretics are usually classified into the following four different groups: thiazides (*e.g.* Hydrochlorothiazide and Bendroflumethiazide), loop diuretics (*e.g.* Bumetanide and Furosemide), potassium-sparing diuretics (*e.g.* Amiloride and Triamterene), and carbonic anhydrase inhibitors (*e.g.* Acetazolamide) according to their chemical structures (see Fig. 1) and physicochemical properties [2–4]. Based on the effect of diuresis, diuretics can also be classified into high-efficiency diuretics (such as loop diuretics), medium-efficiency diuretics (such as thiazides and related compounds) and low-efficiency diuretics (such as potassium-sparing diuretics and carbonic anhydrase inhibitors).

Some methods have been reported for separating and determining diuretics. GC and GC-MS [5, 6] were the traditional methods for the separation and determination of diuretics, and the positive results in the official method of doping control were confirmed by GC-MS. Most diuretics have low volatility and low thermal stability, so when they were separated and detected with GC or GC-MS, derivatization was necessary. As we know, some agents used for derivatization, *e.g.* methyl iodide, have some toxicity, and the process of derivatization was complicated and time-consuming. Several minutes determination may need several days

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Abbreviations: LS, sheath liquid; **TIC**, total ion current

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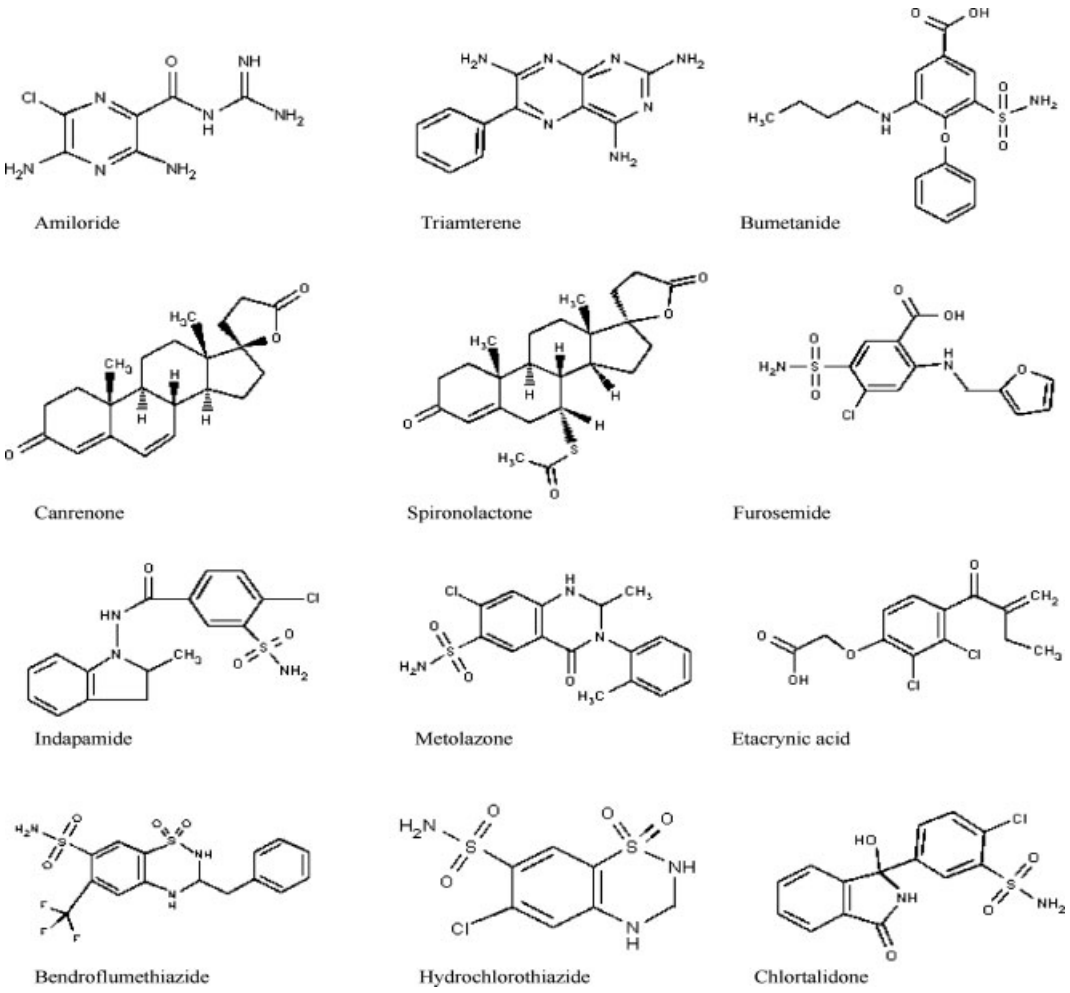

Figure 1. Structures of 12 diuretics

derivatization and pretreatment. Recently, some laboratories tried to use HPLC [7–10] and LC-MS [11, 12] for screening and confirming diuretics. Although LC and LC-MS need not to be tedious in derivatization, they consume longer analysis times and a lot of organic reagents (*e.g.* methanol, ACN). Large volumes of organic reagents are not only expensive, but also contaminate the environment.

Compared to HPLC separation methods, CE offers extremely high separation efficiency, shorter analytical times and lower sample consumption, which is very important for analyzing pharmaceutical compounds in human urine [13–16]. During the Sydney 2000 Olympic Games, about 3000 samples needed to be analyzed in four weeks [11], so for future Olympic Games, a new technology and a fast detection method need to be developed in order to detect a large number of doping samples. CE coupled to UV detection [16], electrochemistry detection [17] and fluorescence detection [18] have been reported for separation and determination of diuretics. These detection methods have some limitations in that UV detection sometimes is not sensitive enough for urine samples. Although electrochemistry and fluorescence detection could provide ultimate sensitivity, they are not suitable for every compound since many analytes do not have electrochemical activity or could not fluoresce. Still some neutral mixtures can not be separated by CZE, such as Spironolactone and Canrenone, when the normal detection methods mentioned above are used. The largest drawback is that the detection method of UV, electrochemistry and fluorescence could not provide information on the structure of the compound, so they are unable to confirm the sample and may be unsuitable for applying to doping control.

MS is a versatile, selective and sensitive detection method, which can give sample structure information. CE-MS is a perfect combination, which combines the high speed, high separation efficiency, simple preparation and very low sample consumption of CE with the high selectivity and sensitivity of MS. A number of CE-ESI-MS methods have been developed for analysis of drugs in urine, such as in clinical and forensic toxicology, in pharmacodynamics and pharmacokinetics [14, 19–21]. CE-MS as a complementary separation method to LC-MS, with extremely high separation efficiency, could be extensively applied in the future [22, 23].

In this paper, 12 diuretics (Amiloride, Triamterene, Bumetanide, Canrenone, Spironolactone, Furosemide, Indapamide, Metolazone, Etacrynic acid, Bendroflumethiazide, Hydrochlorothiazide and Chlortalidone) were studied for the first time by CE-ESI-MS. Under the optimized conditions, ten diuretics, except the neutral compounds Spironolactone and Canrenone, could be separated by CE with DAD at the detection wavelength of 214 nm within 6 min. Using the full-scan mode and SIM scan mode, 12 diuretics could be detected and confirmed by MS. This method could be applied to screen diuretics by DAD and confirm diuretics by MS in real urine samples.

2 Materials and methods

2.1 Chemicals and reagents

Furosemide and Bendroflumethiazide were purchased from Sigma (Sigma-Aldrich, USA). Metolazone was from MP (MP Biomedicals, LLC, Australia). Canrenone was purchased from JK Chemical (Shanghai, China). Amiloride, Bumetanide, Chlortalidone, Etacrynic acid, Indapamide, Spironolactone, Hydrochlorothiazide and Triamterene were obtained from the Chinese Institute of Biological Products Control (Beijing, China). Bumetanide Tablets were provided by Fuzhou Neptunus Fuyao Pharmaceuticals. Hydrochlorothiazide tablets and Triamterene tablets were purchased from Beijing Double-Crane Pharmaceuticals.

ACN, methanol and isopropanol (HPLC grade) were obtained from Sinopharm Chemical Reagents (Shanghai, China). Acetic acid glacial, formic acid, ammonium acetate, ammonium formic, ammonium bicarbonate and ammonium hydroxide were of analytical reagent grade and purchased from Sinopharm Chemical reagents. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2 CE with DAD

CE was carried out on an Agilent ^{3D}CE (Agilent Technologies, Waldbronn, Germany) system with a built-in DAD. All separations were carried out in a 50 μm id bare fused-silica capillary (Agilent Technologies). The total length of the capillary was 57 cm (48.5 cm to the detector). Agilent CE ChemStation was used for the instrument control, date acquisition, and date analysis. The capillary was flushed with water for 10 min, followed by 30 min with 0.1 M NaOH, and then flushed with water for 10 min, finally for 30 min flushed with the running buffer in order to equilibrate the capillary. Between every run the capillary was flushed with water for 3 min and operating electrolyte for 5 min, respectively. The sample was injected with a pressure injection of 0.435 psi for 3 s, and the capillary temperature was set at 25°C. CE-DAD used a shorter capillary compared with CE-MS, so it can provide faster analysis. Initial experiments were carried out with DAD detection to investigate the performance of various electrolytes and other conditions. The CE-DAD method confirms components within a sample by their ultraviolet absorptions and migration times compared with standard materials. Since no structure information of components are provided, CE-DAD can only be used as an initial scan method.

2.3 CE with MS

All CE-ESI-MS experiments were carried out on an Agilent ^{3D}CE (Agilent Technologies) system with a built-in DAD coupled to an Agilent 1100 series single quadrupole mass spectrometor (Agilent Technologies). CE was coupled to MS through an Agilent G1607A orthogonal electrospray interface (Agilent Technologies). A G1603A Agilent CE-MS adapter kit and a G1607A Agilent CE-ESI-MS sprayer kit were used. The CE-MS adapter kit includes a capillary cassette, which facilitates thermostating of the capillary, and the CE-ESI-MS sprayer kit, which simplified coupling the CE system with MS systems, was equipped with an electrospray source. The sprayer had an orthogonal flow design to reduce the detrimental effects caused by the charged particles or droplets, as described by Voyksner and Lee [24]. Electrical contact at the elecrctrospray needle tip was established *via* a liquid sheath flow delivered by an Agilent 1100 series isocratic LC pump (Agilent Technologies). Agilent CE/MSD ChemStation was used for the instrument control, date acquisition, and date analysis.

Analyses were carried out in a bare fused-silica capillary (50 μm id, 90 cm total lenght; Agilent Technologies). Injections were performed hydrodynamically at 0.435 psi for 5 s. DAD detection was by-passed. The capillary temperature was set at 25°C inside the CE-MS capillary cassette (*ca.* 60 cm). The section between the CE instrument and the MS (*ca.* 30 cm) was not thermostated. Before first use, the capillary was conditioned as the capillary in Section 2.2.

MS detection was performed in the ESI positive ionization mode. The electrospray voltage was 3.5 kV for the positive mode in all experiments. MS was operated in full scan mode (partially varied from 200–500 D) and SIM scan mode, simultaneously. The outlet of the capillary was precisely positioned equal with the ESI spray needle through a precise position equipment. Nitrogen was used as the nebulizer gas. The nebulizing gas pressure, the drying gas flow rate and the drying gas temperature were set at 10 psi, 6 L/min and 150°C, respectively. The nebulizing gas pressure at the MS side was set to 0 psi in order to acquire repeatabe quantitative results during the injections, because the high nebulizing gas pressure can lead to sampling error [23, 25]. The fragmentor, step size, and gain were set at 150, 0.15, and 2.0, respectively.

2.4 Preparation of standard and buffer solution

All standard samples were prepared by dissolving corresponding chemicals in methanol at a concentration of 8.70 mmol/L for Amiloride, 1.97 mmol/L for Triamterene,

5.87 mmol/L for Canrenone, 4.80 mmol/L for Spironolactone, 5.47 mmol/L for Metolazone and Indapamide, 5.90 mmol/L for Chlortalidone, 4.75 mmol/L for Bendroflumethiazide, 6.72 mmol/L for Hydrochlorothiazide, 5.48 mmol/L for Bumetanide, 6.60 mmol/L for Etacrynic acid, and 6.05 mmol/L for Furosemide. All buffer stock solutions were prepared conventionally, and the working buffer solutions were prepared by diluting the above stock solutions. All buffers were filtered with a 0.22 μm membrane filter before use. Standard solutions and running buffer were degassed by ultrasonication for 5 min before use.

2.5 Sample preparation

Healthy male volunteers were separated into two groups. One group of volunteers took a single dose of Triamterene tablet (50 mg) orally. The other group of volunteers were administered a single dose of Hydrochlorothiazide (25 mg) and two doses of Bumetanide (2 mg) simultaneously. All doses were administered according to the principle of Public Health Bureau of China. Before administering these drugs, blank urine samples of the volunteers were collected. The urine samples were collected at regular intervals and then stored in the refrigerator at −20°C. All urine samples were filtered through a 0.22 μm membrane filter and then directly sampled, without any other pretreatment.

3 Results and discussion

3.1 CE with DAD detection

Diuretics have been studied by various separation approaches such as CZE [1] and MEKC [26]. These methods have a common feature in that the running buffers used for separation are nonvolatile buffers (*e.g.* sodium phosphate and sodium borate) or contain nonvolatile components (*e.g.* SDS). However, nonvolatile buffers or buffers containing nonvolatile components are not suitable to be applied to CE-MS, because they would contaminate the ion source of MS. It was necessary to find a volatile buffer for CE-MS measurements. Initial experiments were carried out with DAD detection to investigate the performance of various electrolytes and other separation conditions for the analysis 12 diuretics.

Factors influencing the separation and sensitivity for CE, such as type of running buffer, pH, concentration, and separation voltage, were investigated in detail. The types of buffers were first studied. Phosphate and borate are commonly used for CE, but they are not suitable for CE-MS because of their nonvolatility. Formic acid, acetic acid, ammonium formate, ammonium acetate, ammonium bicarbonate, and ammonium hydroxide can be used for CE-MS. Ten diuretics (except two neutral compounds) were first studied in the volatile buffers and the experiments showed that better separation of these diuretics could be obtained with ammonium formate buffer.

The pH of the buffer was very important for the separation efficiency because the pH of the buffer medium affected the EOF. In our experiments, the separation efficiency was found to be increased with the pH of the buffer solution in the range of 3.0–9.0 (see Fig. 2). Some components gave no signal by DAD in the lower pH, range and when pH = 10.0, resolution between peaks 4 and 5, also between peaks 6 and 7,

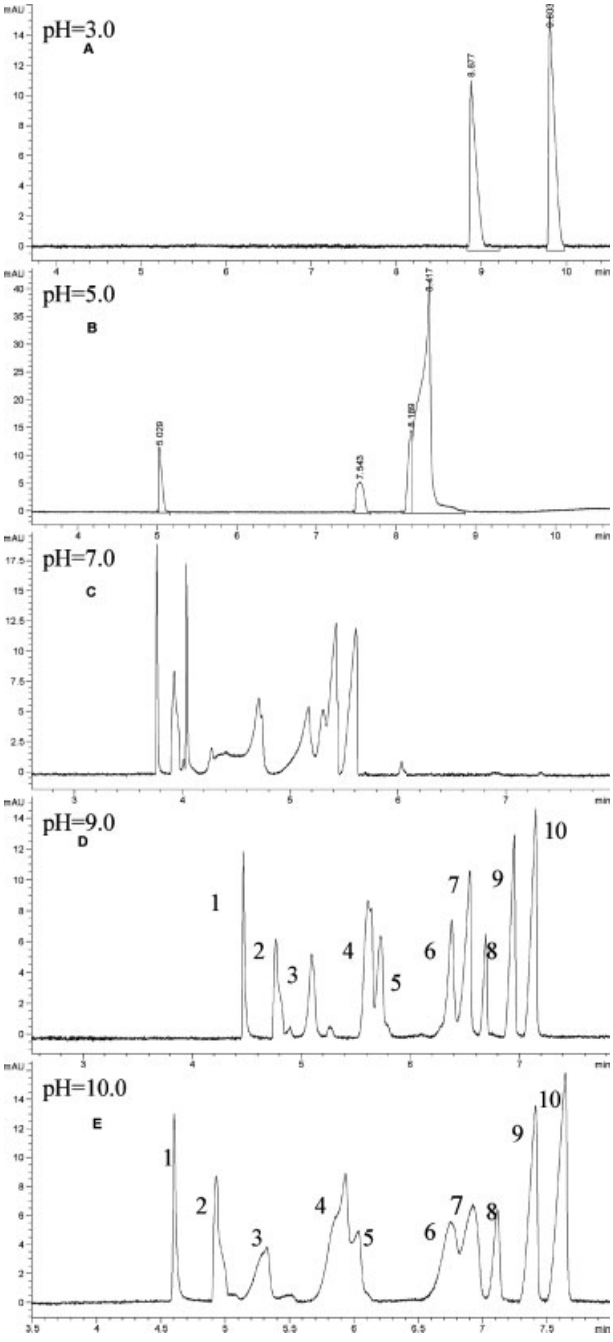

Figure 2. Effect of pH on the separation of the diuretics. Bare fused-silica capillary: 50 μm id and 48.5 cm effective length; injections: 0.435 psi × 5 s; detection wavelength: 214 nm; separation voltage: 18 kV; running buffer: 40 mM ammonium formate.

became poor. Therefore, more detailed investigations between pH 9.0 and 10.0 were carried out. Experiments indicated that better separation of ten diuretics was achieved at pH 9.40. The separation in CZE is based on the ratio of *m/z* of the compounds. Thus, Triamterene, Spironolactone and Canrenone can not be separated by CZE because they are neutral in the applicable pH range.

The effect of concentration of the buffer on separation was tested from 10 to 60 mM. As anticipated, better separation of the diuretics was obtained at higher concentrations of the buffer. Some components could not be separated at low concentrations of the buffer. However, high concentration of the buffer (exceeding 50 mM) would result in large background current at the same voltage, which would give higher Joule heat, leading to a poor repeatability. When the concentration of ammonium formate was 40 mM, both higher efficiency and better peak shape were obtained.

The separation voltage was also investigated. In our experiment, the analysis time was prolonged at low voltage, but the resolution was not improved. In order to obtain better resolution and shorter analytical times, a 20 kV separation voltage was adopted. The optimum wavelength for detection of these compounds was examined in the range of 190–350 nm, and the results showed that 214 nm was the most suitable wavelength for this method to achieve the best sensitivity.

To sum up, the optimum conditions for separating the above mentioned compounds can be described as follows: 40 mM ammonium formate at pH 9.40 as the running buffer solution, and 20 kV as separation voltage, 5 s and 0.435 psi as injection conditions. Under these conditions, a typical electropherogram for a standard mixture solution with the ten diuretics is shown in Fig. 3. It was clear that there was baseline separation of the ten analytes within 6 min with CE.

In order to investigate the sensitivity of the DAD detection system, a series of standard samples comprising ten diuretics were tested under the above described optimum conditions. The linear responses of ten diuretics were observed over the range 4.75–435 μmol/L (see Table 1). All coefficients were >0.99. On the basis of

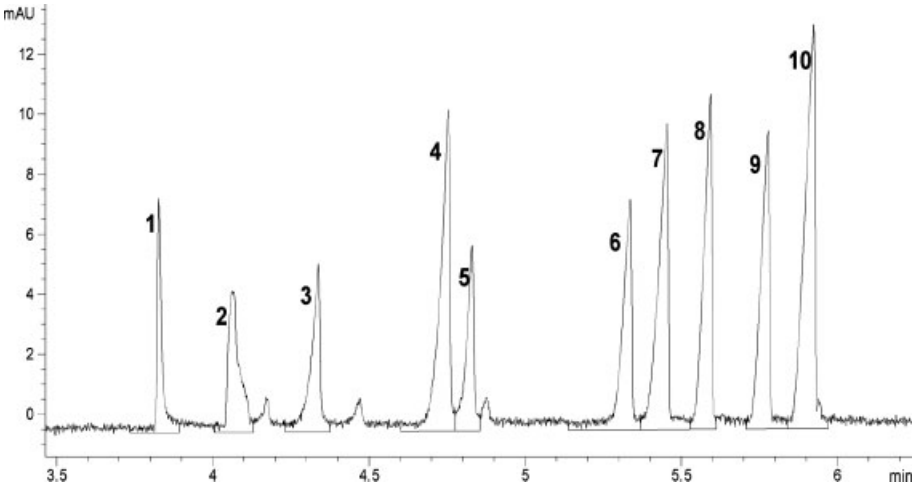

Figure 3. CE-DAD electropherogram of a standard mixture of ten diuretics in the optimized conditions. 1. Amiloride (435 μmol/L); 2. Triamterene (395 μmol/L); 3. Metolazone (273 μmol/L); 4. Chlortalidone (295 μmol/L); 5. Indapamide (273 μmol/L); 6. Bendroflumethiazide (237 μmol/L); 7. Hydrochlorothiazide (336 μmol/L); 8. Bumetanide (274 μmol/L); 9. Etacrynic acid (330 μmol/L); 10. Furosemide (302 μmol/L). Separation voltage: 20 kV; running buffer: 40 mM ammonium formate (pH 9.40); other conditions were the same as in Fig. 2.

Table 1. Regression equations, linearity and the detection limits of CE-DAD[a)]

Compound	Regression equation	R^2	Linear range (μmol/L)	Detection limit (μmol/L)
Amiloride	$y = 0.1140x - 0.7921$	0.9984	8.71–435	2.31
Triamterene	$y = 0.0856x - 1.4255$	0.9960	7.90–395	2.54
Metolazone	$y = 0.0833x + 0.0901$	0.9943	5.47–273	1.76
Chlortalidone	$y = 0.2045x + 1.0097$	0.9956	5.90–295	1.28
Indapamide	$y = 0.1603x + 1.5662$	0.9994	5.47–273	1.66
Bendroflumethiazide	$y = 0.1621x - 0.4744$	0.9947	4.75–237	1.47
Hydrochorothiazide	$y = 0.1395x + 1.3145$	0.9982	6.72–336	1.38
Bumetanide	$y = 0.1933x - 0.2611$	0.9973	5.48–274	1.23
Etacrynic acid	$y = 0.1197x + 0.4005$	0.9985	6.60–330	2.07
Furosemide	$y = 0.1758x + 0.0263$	0.9949	6.05–302	1.42

a) Bare-fused silica capillary: 50 μm id and 48.5 cm effective length; injections: 0.435 psi × 5 s; detection wavelength: 214 nm; separation voltage: 20 kV; running buffer: 40 mM ammonium formate (pH 9.40).

S/N = 3, the DAD detection limits of ten diuretics were obtained in the range of 1.23–2.54 μmol/L. The results of regression analysis on calibration curves, linearity and detection limits are presented in detail in Table 1.

3.2 CE with MS detection

Directed hyphenation of CE to MS was adopted in this experiment by an orthogonal electrospray interface with a triple-tube sheath liquid (LS) arrangement. Electrospray ionization is the ideal method for online interfacing of CE to MS because of the facilitation of the transfer of the analytes from the liquid phase of the CE to the gas phase of the MS [27]. Orthogonal electrospray interface has the benefit of improving system cleanliness which allows the greatest flexibility in the choice of CE buffer salts, concentrations and additives. The separation capillary is surrounded by the second tube of a larger diameter in a coaxial arrangement. The supportive liquid is guided through this outer tube and mixes with the CE buffer directly in the taylor cone. The outside tube is used for nebulization gas to support droplet formation. The triple tube interface systems are simple and provide the possibility to overcome the limitations in the electrospray compatibility of the CE buffer.

BGE and LS may significantly influence the CE-MS separation since the ionic boundary was formed when different BGE and LS were used [28]. Therefore, the type of LS, the flow rates of LS and the flow rates of drying gas were first investigated.

Experiments indicated that the selection of LS was very important for the separation efficiency and sensitivity. Methanol-water and isopropanol-water were chosen to be the LS in our experiment, and the results are shown in Fig. 4. It showed that isopropanol was much better than methanol, as isopropanol tended to yield the strong and stable signals for the analysis of diuretics. The proportion of isopropanol and water was further investigated, and the results were illustrated in Fig. 5, which indicated that the ratio of 1:1 v/v for isopropanol-water could provide the best compromise with good signal stability and sensitivity. Adding 30 mM acetic acid to the LS could provide an excellent separation and sensitivity.

The experiment showed that a LS flow rate of 4 μL/min was sufficient to obtain a stable spray with minimal dilution of CE effluent to achieve high sensitivity. The drying gas flow was kept as low as possible to avoid problems with aspirating liquid through the CE capillary or pressurizing the source region. Stable spray could be achieved at a drying gas flow rate of 10 psi, 6 L/min and a drying gas temperature of 150°C.

The MS detections were carried out in the positive mode with both full scan and SIM scan mode. The total ion current (TIC) CE-MS chromatogram scan range was 200–500 *m/z*. Experiments showed that some of the diuretics produced an $[M+H]^+$ ion or $[M+NH_4]^+$ ion, and others of the diuretics

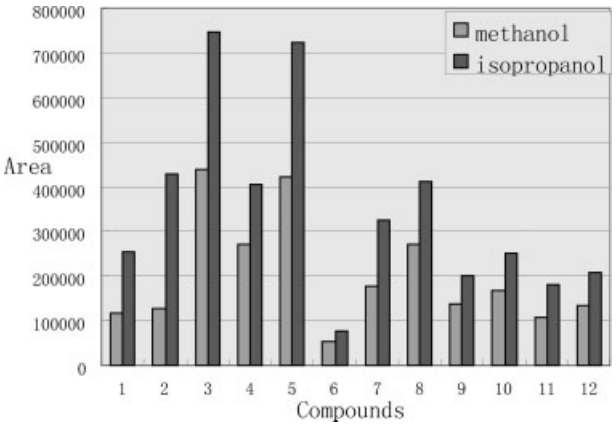

Figure 4. Influence of LS composed of methanol and isopropanol on peak area. Bare fused-silica capillary: 90 cm × 50 μm; injections: 0.435 psi × 5 s; separation voltage: 20 kV; running buffer: 40 mM ammonium formate (pH = 9.40); nebulizing gas pressure: 10 psi; the drying gas flow rate: 6 L/min; drying gas temperature: 150°C. 1. Amiloride; 2. Triamterene; 3. Canrenone; 4. Spironolactone; 5. Metolazone; 6. Chlortalidone; 7. Indapamide; 8. Bendroflumethiazide; 9. Hydrochlorothiazide; 10. Bumetanide; 11. Etacrynic acid; 12. Furosemide

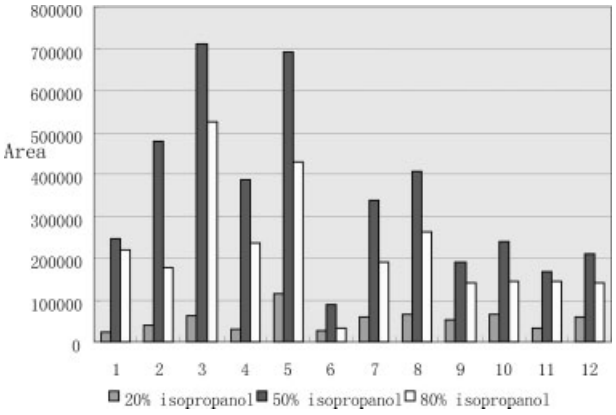

Figure 5. Influence of the proportion of the isopropanol in the LS on peak area. Other conditions were the same as in Fig. 4.

formed an $[M+H]^+$ ion and an $[M+NH_4]^+$ ion (see Table 2), because all mass spectrum measurements were carried out in the ammonium environment. In order to obtain highly sensitive and simultaneous detection $[M+H]^+$ ion and $[M+NH_4]^+$ ion were adopted. Under the optimized conditions, 12 diuretics could be separated within 20 min and confirmed overall by their characteristic ions (see Fig. 6).

Isomer Metolazone and Indapamide were electrophoretically separated completely with migration times of 4.337 and 4.828 min, respectively (see Table 2). Because the length of the capillary for MS (90 cm) was longer than that for CE (48.5 cm), the migration times of Metolazone and Indapamide were 13.162 and 14.961 min (see Table 2) in the CE-MS mode. The full resolution made isomers to be

Table 2. Migration time of DAD, migration time of MSD and characteristic ions in the ESI for twelve diuretics[a)]

Compound	M_t[c)]DAD (min)	M_t[c)] MS (min)	Characteristic ions of ESI	
			$[M+H]^+$(R[b)])	$[M+NH_4]^+$(R[b)])
Amiloride	3.828	11.563	230.0	
Triamterene	4.064	12.448	254.0	
Canrenone	4.064	12.448	341.1 (100)	358.3 (20)
Spironolactone	4.064	12.448	417.2 (30)	434.2 (100)
Metolazone	4.337	13.163	366.1	
Chlortalidone	4.752	14.690		356.0
Indapamide	4.828	14.961	366.1	
Bendroflumethiazide	5.335	16.853		439.1
Hydrochlorothiazide	5.452	17.301		315.0
Bumetanide	5.592	17.831	365.2	
Etacrynic acid	5.776	18.644	303.0 (40)	320.1 (100)
Furosemide	5.992	19.169	331.0 (20)	348.0 (100)

a) DAD and MSD conditions were as shown in Fig. 3 and Fig. 6 respectively.
b) Relative intensity.
c) Migration time.

selectively detected at their $[M+H]^+$ ions with m/z of 366.1 by the mass spectrometer. In this system, the isomers Metolazone and Indapamide were both identified and quantified by CE-ESI-MS.

Triamterene, Spironolactone and Canrenone could not be separated in CZE because of their neutral characteristics, however, both qualitative and quantitative information could be obtained by extracting their characteristic ions from TIC electropherograms. Under the above optimum conditions, the characteristic ion of Triamterene was $[M+H]^+$ with m/z of 254.1, the characteristic ion of Spironolactone was $[M+NH_4]^+$ with m/z of 434.2, and the characteristic ion of Canrenone was $[M+H]^+$ with m/z of 341.1 (see Table 2). As shown in Fig. 6, number 2, 3 and 4 are the extracted characteristic ion electropherograms for Triamteren, Spironolactone and Canrenone, respectively.

3.3 CE-MS validity

In order to investigate the quantification of the system, a series of standard of twelve diuretics mixture solution with concentration levels from 0.43 to 1088 μmol/L were tested using the SIM scan mode under optimum conditions (see Section 3.2). As mentioned in Section 1, Spironolactone and Canrenone can not be separated by CZE; in this study the extracted ion means was adopted for quantitation. The calibration curves of 12 diuretics exhibited linear dynamic ranges with correlation coefficients (R^2) in the range of 0.9921–0.9978. On the basis of S/N = 3, the mass detection limits of 12 diuretics were obtained and they were in the range of 0.13–2.7 μmol/L. The results of limits and regression analysis on calibration curves and detection limits are presented in detail in Table 3.

To examine the precision, the repeatability of the method was measured by five injections of a standard mixture solution. The detective results were represented in Table 4, the RSDs of peak areas were 1.6–8.8% and the RSDs of migration times were 0.24–0.94%.

Usually, when urine samples are directly introduced into the capillary, some protein components can be then fixed to the capillary wall producing irreproducibility in migration time. However, between runs the capillary was only conditioned with water and buffer in our experiment. Contrary to other works, in this paper, a good repeatability was obtained. Based on the review "Capillary electrophoresis of diuretics" written by Riekkola, M.-L. and Jumppanen, J. H. (see [1]), the proteins and large peptides can be easily adsorbed onto the surface of the silica capillary wall when the pH is above 2 and less than the pI of the protein or the peptide. The urine protein is mainly albumin, and the pI of albumin is between 4.6 to 4.7. The pIs of most proteins in urine are lower than 6. The pH of the buffer in the experiments was 9.40, which is higher than the pI of proteins in urine, so this may probably be the reason that the experiments could obtain good repeatability.

CE-ESI-MS detection was also employed for the determination of spiked samples recoveries (see Table 5). For this purpose, known amounts of diuretics were added to a spiked urine sample containing different concentrations of the 12 diuretics. Recoveries were determined *via* comparison of the mean ($n = 3$) of the peak areas that were obtained through experiments with those added to the urine. The recoveries of the 12 diuretics were found to be between 72.4 and 118%. The RSDs of recoveries for the 12 diuretics were lower than 10%.

Table 3. Regression equations, linearity and the detection limits of CE-ESI-MS[a)]

Compound	Regression equation	R^2	Linear range (μmol/L)	Detection limit (μmol/L)
Amiloride	$y = 6811.7x - 6820.8$	0.9941	0.43–87	0.13
Triamterene	$y = 2091.6x - 2473.5$	0.9921	0.63–95	0.20
Canrenone	$y = 1650.3x - 4571.7$	0.9947	2.6–188	0.85
Spironolactone	$y = 246.35x - 258.74$	0.9940	2.1–154	0.70
Metolazone	$y = 4046.2x + 11675$	0.9966	1.8–328	0.55
Chlortalidone	$y = 1337.0x + 26068$	0.9931	5.9–850	1.5
Indapamide	$y = 4339.2x + 6672.4$	0.9978	1.8–210	0.57
Bendroflumethiazide	$y = 2716.2x + 11553$	0.9940	4.7–546	1.6
Hydrochorothiazide	$y = 648.70x + 660.12$	0.9943	8.7–517	2.7
Bumetanide	$y = 7058.2x + 19288$	0.9974	0.9–165	0.33
Etacrynic acid	$y = 961.17x + 11476$	0.9947	6.6–1188	2.3
Furosemide	$y = 578.96x + 14010$	0.9948	6.0–1088	2.0

a) CE-MSD conditions as in Fig. 6.

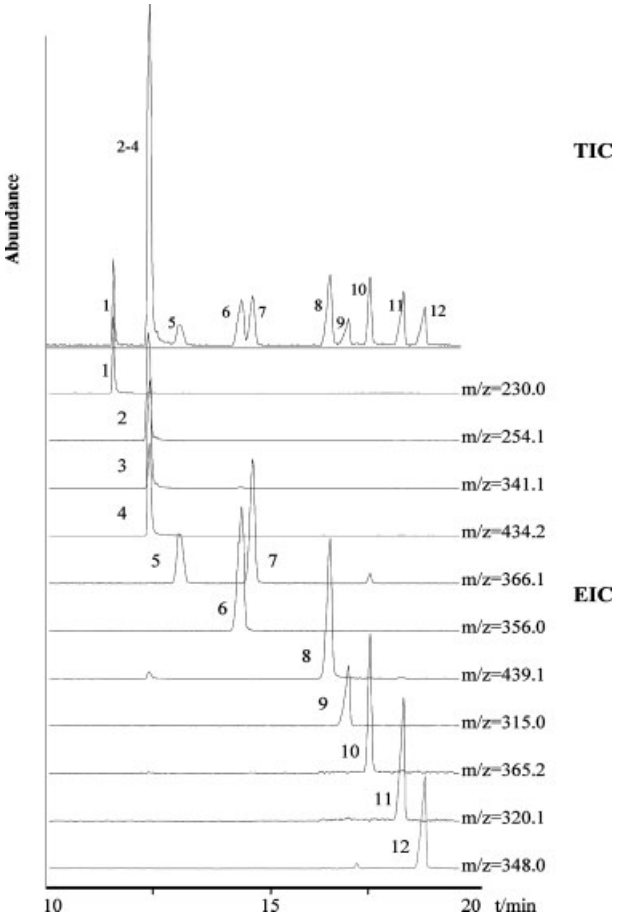

Figure 6. TIC and extracted ion electropherograms of 12 diuretics. LS: 4 μL/min, 30 mM acetic in 50% v/v isopropanol-water; other conditions as in Fig. 4.1. Amiloride; 2. Triamterene; 3. Canrenone; 4. Spironolactone; 5. Metolazone; 6. Chlortalidone; 7. Indapamide; 8. Bendroflumethiazide; 9. Hydrochlorothiazide; 10. Bumetanide; 11. Etacrynic acid; 12. Furosemide.

Table 4. Repeatability of CE-MSD[a)]

Compound	Concentration (μmol/L)	RSD ($n = 5$) (%)	
		Migration time	Peak area
Amiloride	25.28	0.41	5.5
Triamterene	34.11	0.50	4.6
Canrenone	67.67	0.71	3.4
Spironolactone	55.31	0.68	2.2
Metolazone	94.47	0.24	8.8
Chlortalidone	306.1	0.24	8.2
Indapamide	94.47	0.35	1.6
Bendroflumethiazide	164.0	0.92	1.6
Hydrochorothiazide	348.3	0.94	6.9
Bumetanide	47.32	0.42	6.2
Etacrynic acid	342.1	0.28	3.4
Furosemide	313.5	0.41	8.6

a) CE-MSD conditions as in Fig. 6.

3.4 CE-MS analysis of the diuretics in urine

In this paper, direct injection and online detection methods were applied to the determination of diuretics in real urine samples. As most diuretics were excreted unchanged in the human urine, it was possible to screen and confirm them by focusing on the direct detection of the unchanged diuretics from the human urine [11, 12]. According to Section 2.5, the excretions of the group Triamterene tablet (50 mg) and the other group of Hydrochlorothiazide (25 mg) and Bumetanide (2 mg) were simultaneously studied in this experiment. Since Hydrochlorothiazide can be synergetic, Hydrochlorothiazide (25 mg) was selected to be taken orally with Bumetanide (2 mg) in this experiment. The real urine samples

Table 5. Recoveries of spiked samples[a)]

Sample	Compound	Added (μmol/L)	Found (μmol/L)	Recovery (%)	RSD (%) (n = 3)
Urine	Amiloride	8.71	7.62	87.5	4.90
		34.8	35.3	101	6.02
	Triamterene	7.90	6.77	85.7	7.33
		31.6	31.0	98.0	6.80
	Canrenone	17.6	13.0	73.9	8.53
		70.5	62.6	88.8	7.42
	Spironolactone	12.0	12.6	105	7.50
		48.0	46.8	97.4	8.12
	Metolazone	27.3	25.1	91.9	6.92
		109	98.2	90.0	5.88
	Chlortalidone	44.3	47.7	108	9.86
		177	186	105	9.18
	Indapamide	19.1	16.4	85.9	7.68
		76.5	67.2	87.8	8.29
	Bendroflumethiazide	47.5	45.9	96.6	8.14
		190	223	118	6.32
	Hydrochlorothiazide	67.2	51.7	76.9	5.85
		269	217	80.9	7.19
	Bumetanide	13.7	15.4	112	9.36
		54.8	56.9	104	8.40
	Etacrynic acid	66.0	55.2	83.6	6.32
		264	238	90.2	5.98
	Furosemide	60.5	43.8	72.4	6.17
		242	215	89.0	4.66

a) CE-MSD conditions as in Fig. 6.

were injected directly only with simple filtration to CE at a pressure of 0.435 psi for 5 s, corresponding to an injection volume of about 3.0 nL [23].

Under the optimal conditions of CE-ESI-MS, Triamterene, Hydrochlorothiazide and Bumetanide in the authentic urine samples were detected based on the migration times and characteristic ions. Figure 7 illustrates the variation of the concentration of Triamterene with regular interval excretion times of urine, Fig. 7A was the TIC electropherogram of excretion time of urine, and Fig. 7B was the concentration-time profile. As shown in Fig. 7A, no interference was observed between the endogenous matter in the authentic urine samples and the target compound due to the migration times. In Fig. 7B, it could be seen that the excreted time of the maximum excretion concentration was 2 h when the excreted time was in the range of 0–10 h. Figure 7B therefore clearly reflected the metabolic process of Triamterene in the urine.

In the case of the real urine sample of Hydrochlorothiazide and Bumetanide, Fig. 8 proved the presence of Hydrochlorothiazide (m/z 315.0, $[M+NH_4]^+$, see Fig. 8E) and Bumetanide (m/z 365.2, $[M+H]^+$, see Fig. 8F). Figure 8A was the TIC electropherograms of the blank urine sample; Fig. 8B was the TIC electropherograms excretion urine sample of Bumetanide and Hydrochlorothiazide. Figures 8C and D were the extracted ion electropherograms of Bumetanide and Hydrochlorothiazide and Figs. 8E and F were the mass spectra of Bumetanide and Hydrochlorothiazide. The real urine sample was obtained from a healthy men after oral administration of Hydrochlorothiazide (25 mg) and Bumetanide (2 mg). The experimental results showed that the CE-MS detection system could be used successfully for confirmation purposes of diuretics.

4 Concluding remarks

We have developed a rapid, simple and reproducible CE-ESI-MS method for the simultaneous determination of diuretics. Compared with other techniques, this new method has several advantages: (i) diuretics can be directly analyzed without tedious derivatization; (ii) the analysis is extremely fast and simple; (iii) real urine samples only need simple filtration pretreatment; and (iv) micro-amounts of sample and support solution do not contaminate the environment. In fact, diuretics are screened fast by DAD, within 6 min, and determined in less than 20 min by MS with no pretreatment for real urine samples. Furthermore, the present methodology provides good reproducibility and linearity, and excellent

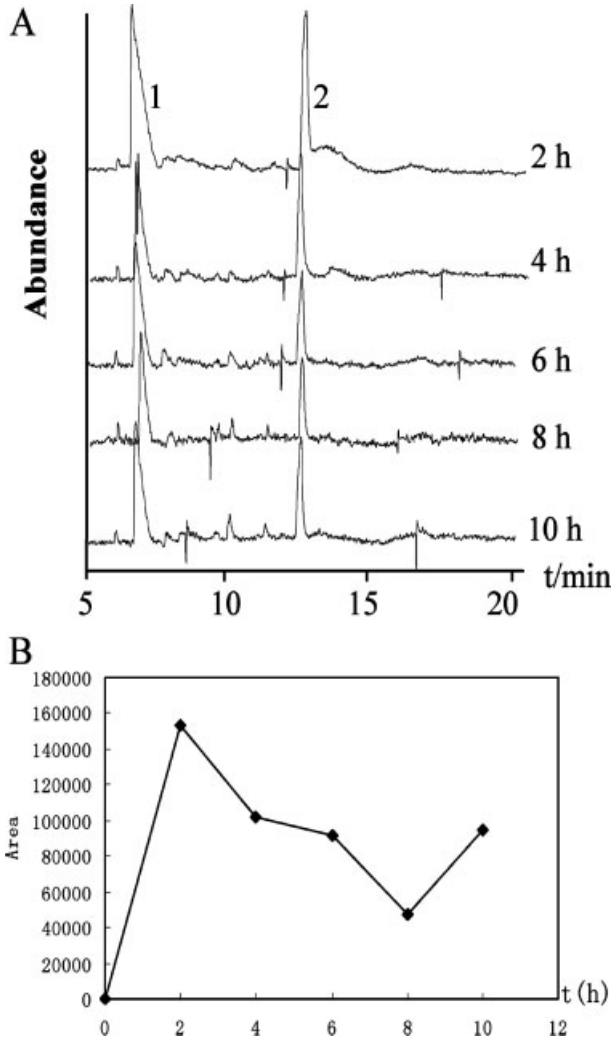

Figure 7. TIC electrograms (A) and concentration-time profile obtained from human urine 2, 4, 6, 8 and 10 h after the oral administration of triamterne. CE-MS detection conditions as in Fig. 6. 1. Endogenous matter; 2. Triamterne.

identification capability. Its utility will provide double assurance in doping controls with fast scan by DAD and confirmation by MS, which will decrease the rate of false positives in doping controls. The CE-MS technique could be broadly applied in doping control and could contribute to the anti-doping as a simple, robust, rapid method.

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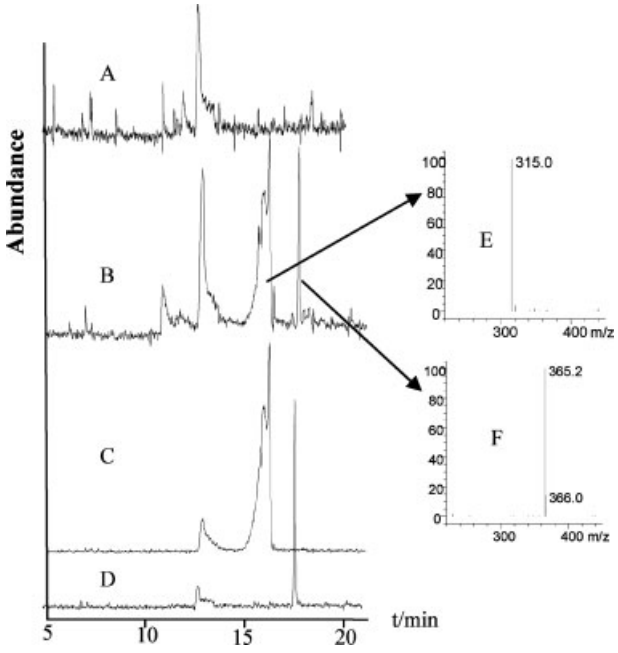

Figure 8. TIC electrograms of blank (A), the TIC electrograms (B), extracted ion electropherograms (C, D) and mass spectra (E, F) obtained from human urine after the oral administration of Hydrochlorothiazide and Bumetanide. CE-MS detection conditions as in Fig. 6.

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